Changes in the ghrelin hormone pathway maybe part of an unusual gastric system in monotremes

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Abstract

Ghrelin is a growth hormone (GH)-releasing and appetite-regulating peptide predominately released from the stomach. Ghrelin is evolutionarily highly conserved and known to have a wide range of functions including the regulation of metabolism by maintaining an insulin-glucose balance. The peptide is produced as a single proprotein, which is later proteolytically cleaved. Ghrelin exerts its biological function after *O*-n-octanoylation at residue serine 3, which is catalyzed by ghrelin *O*-acyl transferase (GOAT) and allows binding to the growth hormone secretagogue receptor (GHS-R 1a). Genes involved in the ghrelin pathway have been identified in a broad range of vertebrate species, however, little is known about this pathway in the basal mammalian lineage of monotremes (platypus and echidna). Monotremes are particularly interesting in this context, as they have undergone massive changes in stomach anatomy and physiology, accompanied by a striking loss of genes involved in gastric function. In this study, we investigated genes in the ghrelin pathway in monotremes. Using degenerate PCR, database searches and synteny analysis we found that genes encoding ghrelin and GOAT are missing in the platypus genome, whilst, as has been reported in other species, the *GHSR* is present and expressed in brain, pancreas, kidney, intestine, heart and stomach. This is the first report suggesting the loss of ghrelin in a mammal. The loss of this gene may be related to changes to the platypus digestive system and raises questions about the control of blood glucose levels and insulin response in monotreme mammals. In addition, the conservation of the ghrelin receptor gene in platypus indicates that another ligand(s) maybe acting via this receptor in monotremes.

Key words

Ghrelin; GOAT; GHS-R 1a; Monotremes; Platypus; Evolution; Metabolic control

1. [*]Introduction

1.1 Ghrelin pathway

Ghrelin is a 28 amino acid peptide and endogenous ligand for the growth hormone secretagogue receptor (GHS-R 1a)[†] (Kojima et al., 1999). It was first purified from the rat stomach by Kojima *et al.* based on its GHS-R 1a stimulating and growth hormone (GH) releasing activities (Kojima et al., 1999), but has since been shown to be expressed in all tissues investigated so far, including pancreatic epsilon cells (ε cells) (Wierup et al., 2002) and gastric P/D_1 cells (known as X/A-like cells in rodents) (Date et al., 2000). In a variety of vertebrate species, ghrelin is known to be involved in a range of activities, not only the stimulation of GH secretion but also food intake, regulation of glucose and lipid metabolism, increasing gastric acid release and motility, decreasing blood pressure and various neuronal functions (Kojima and Kangawa, 2008; Kaiya et al., 2012).

The human ghrelin gene (*GHRL*) encodes a preproghrelin protein, which gives rise to ghrelin and obestatin via posttranslational proteolytic cleavage (Zhang et al., 2005). Acylation at its third serine residue (Ser^3) enables the bidirectional transport of ghrelin across the blood-brain barrier (Banks et al., 2002) and is essential for GHS-R 1a binding and consequent biological activities (Kojima et al., 1999). However, acyl ghrelin only makes up ~25% of the total circulating ghrelin and the majority is nonacylated (des-acyl ghrelin). Although the specific receptor for des-acyl ghrelin is not yet known, some studies have reported effects on food intake, energy expenditure and glucose homeostasis (reviewed by (Kirchner et al., 2012)). Ghrelin *O*-acyl transferase (GOAT), which belongs to the membrane-bound *O*-acyl transferase (MBOAT) family and is encoded by *MBOAT4*, is the only enzyme known to catalyse the acylation of ghrelin. Moreover, the recognition sequence of GOAT (GXSFX,

Abbriations: GH, growth hormone; GHRL, ghrelin; GHS-R growth hormone secretagogue receptor; GOAT Ghrelin O-acyl transferase; GPCR: G-protein coupled receptor; MBOAT membrane-bound O-acyl transferase.

where X is any residue) is specific for ghrelin, indicating ghrelin is the only substrate of GOAT (Yang et al., 2008b; Ohgusu et al., 2009). Murine GOAT is mainly expressed in the stomach, but is also found in other tissues including pancreas, small intestine and colon (Kirchner et al., 2009; Gutierrez et al., 2008; Yang et al., 2008a). *MBOAT4* mRNA is expressed mostly by ghrelin-producing X/A-like cells of the gastric oxyntic mucosa in mice (Sakata et al., 2009).

The ghrelin receptor, GHS-R 1a, is a 7 trans-membrane G-protein coupled receptor (GPCR), encoded by the *GHSR.* Alternative splicing of *GHSR* creates two isoforms in humans: the biologically functional GHS-R 1a and the truncated GHS-R 1b (Howard et al., 1996). GHS-R 1b does not bind ghrelin, but forms dimers with GHS-R 1a to inhibit its function (Leung et al., 2007) and expression (Chow et al., 2012). *GHSR* is predominately expressed in the hypothalamus and pituitary gland of the central nervous system (CNS) (Guan et al., 1997) but is also found in peripheral tissues such as the pancreas and spleen (Gnanapavan et al., 2002). High basal signaling activities have led to the idea that GHS-R 1a can also act independently of ghrelin (Holst et al., 2003).

The ghrelin pathway is vital to maintaining growth hormone (GH) release and energy homeostasis. Orthologs of genes in this pathway, including *GHRL*, *GHSR* and *MBOAT4*, are highly conserved and have been identified in a range of vertebrate species (Kojima et al., 1999; Tanaka et al., 2001; Kaiya et al., 2008; Kaiya et al., 2009; Gutierrez et al., 2008; Kojima and Kangawa, 2005).

1.2 The stomach and pancreas of monotremes

The small and glandless monotreme stomach is one of the most striking anatomical differences between the monotreme lineage and other mammalian species. The stomach of platypus (*Ornithorhynchus anatinus*) and echidna (*Tachyglossus aculeatus*) are lined with a

stratified squamous epithelium that lacks fundic glands. The only glands present are the Brunner's glands, which are confined to the submucosa of the distal stomach (Krause, 1971; Griffiths, 1978; Krause and Leeson, 1974).

The platypus genome has been sequenced recently providing important insights into mammalian evolution and into the extraordinary biology of monotremes (Warren et al., 2008). A key feature of the genome analysis of the platypus was the identification and characterization of genes involved in protein degradation (degradome). This revealed the wholesale loss of genes required for gastric function, including genes encoding gastrin (*GAST*) and pepsin (*PGA, PGC*) (Ordonez et al., 2008). The loss of these genes is consistent with the striking physiological and anatomical changes of the platypus digestive tract but raised questions about other genes and pathways related to digestion and metabolic control involving other organs (e.g. the pancreas) in platypus.

Very little is known about the anatomical structure and function of the platypus pancreas although the echidna pancreas has shown to have distinctive endocrine and exocrine parts (Yamada et al., 1990). Similar to other mammals, the echidna's endocrine islets of Langerhans contain α, β, δ and PP cells (Yamada et al., 1990). However, the existence of ghrelin-producing ε cells in monotremes has not been reported.

Here we investigated genes in the ghrelin pathway in monotremes, which have undergone massive changes in stomach anatomy and physiology accompanied by attrition of the degradome gene repertoire. Our findings suggest that genes encoding ghrelin/obestatin and GOAT are missing in the platypus genome, whilst *GHSR* is present and is expressed in brain, pancreas, intestine, kidney, heart and stomach. These findings raise important questions about the ghrelin pathway and metabolic control in this mammalian lineage.

2. Materials and Methods

2.1 Degenerate PCR

Primers were designed using NCBI primer-blast online program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were synthesized at Geneworks (Adelaide, SA, Australia). For *GHRL* degenerate primers (Table 1 NO. 1-7) were designed to match the most conserved regions identified by multiple coding sequences (cds) alignments among human (*Homo sapiens*) NM_001134941.1, mouse (*Mus musculus*) NM_021488.4, opossum (*Monodelphis domestica*) XM_001375640.2, chicken (*Gallus gallus*) NM_001001131.1, cow (*Bos taurus*) NM_174067.2 and rabbit (*Oryctolagus cuniculus*) XM_002722463.1 (Supplementary Fig. 1).

For *MBOAT4* degenerate primers (Table 1 NO. 8-10) were designed by multiple coding sequence (cds) alignments among human (*Homo sapiens*) NM_001100916.1, mouse (*Mus musculus*) NM_001126314.2, opossum (*Monodelphis domestica*) XM_001372794.2, chicken (*Gallus gallus*) NM_001199289.1 cow (*Bos taurus*) NM_001192257.1 and rabbit (*Oryctolagus cuniculus*) XM_002709539.1 (Supplementary Fig. 1).

Genomic DNA was isolated from platypus, echidna, mouse, opossum and chicken liver tissue using phenol/chloroform/isoamyl alcohol (Sigma Aldrich, USA) method as described in (Strauss, 2001). All PCRs (for *GHRL*, with primer pairs 1&4, 6&7, 2&5, 2&3, 1&3 and for *MBOAT4* with primer pairs 8&10 and 9&10) were performed (and repeated at least twice) in a total volume of 25 μl containing 100 ng genomic DNA, 1X PCR reaction buffer (Promega) and final concentrations of dNTPs (0.1 mM), each primer (0.4 μM) and Taq polymerase (0.012 U/μl). Reactions were carried out on a PTC-200 DNA Engine thermal cycler (MJ Research) using the following parameters: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C (except for primer pairs 9 & 10 where annealing was done at 45°C) for 30 s, and extension at 72°C for 1 min, followed by a

final extension at 72°C for 7 min. PCR products were checked on a 1.5% agarose gel. The identity of all PCR products was confirmed by DNA sequencing.

2.2 RNA extraction

Platypus tissues were obtained from an adult platypus (Animal ethics permits AEEC R.CG.07.03 and AEC S-49-200 to F.G). Total RNA was extracted from snap frozen platypus tissues (brain (frontal cortex), pancreas, liver, lung, intestine (proximal small intestine), stomach, heart and kidney) using TRIzol reagent (Invitrogen, USA) according to the manufacture's instructions. RNA was resuspended in nuclease free water and stored at -80 °C.

2.3 cDNA synthesis

cDNA was synthesized from 3 μg RNA with Superscript III Reverse Transcriptase (Invitrogen) following the manufacture's instructions. Briefly, RNAs were treated with DNase I (Roche) to remove genomic DNA, incubated with 50 ng of random hexamers and 0.5 μl of 10 mM dNTPs for 5 min at 65°C. After incubation, 2 μl of 5X First-strand RT buffer, 0.5 μl of 0.1 M dithiothreitol (DTT), 0.5 μl of RNaseOUT™ (40 U/μl), and 0.5 μl SuperScript III Reverse Transcriptase (200 U/μl) were added and incubated at 25°C for 10min, and then 50°C for 50 min, followed by the final termination at 85°C for 5 min. Finally, 0.2 μl of RNase H (Biolabs, 5 U/μl) were added to each tube and incubated at 37°C for 20 min. cDNAs were stored at −20°C.

2.4 RT-PCR

RT-PCR was performed to determine the presence of *GHSR* mRNA in different platypus tissues. Two gene-specific primers (NO. 11 & 12 in Table 1) were designed based on the known coding sequence of platypus *GHSR*. Amplification cycles were: initial denaturation at 94°C for 3min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for

30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. PCR products were resolved on 1.5% agarose gels and then visualized with ethidium bromide. The identity of all PCR products was determined by DNA sequencing.

2.5 Database searches

Gene and contig information for multiple alignments physical location and synteny analysis were acquired from ENSEMBL genome browser (http://www.ensembl.org/). BLAST searching including Trace Archive Nucleotide BLAST was performed by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/), UCSC (http://genome.ucsc.edu/) and also transcriptome databases ViroBLAST (Deng et al., 2007) and ISA modules (Brawand et al.). Sequence alignments were conducted using the ClustalW algorithm (Thompson et al., 1994). Synteny analysis was done by searching the UCSC genome browser (http://genome.ucsc.edu/index.html) for the following species: human (*Homo sapiens,*), opossum (*Monodelphis domestica*), chicken (*Gallus gallus*), anole lizard (*Anolis carolinensis*) and platypus (*Ornithorhynchus anatinus*). Phylogenetic trees were built using the MacVector v11.0.4 software package (Cary, NC, USA). The ratio (dS/dN) of synonymous (dS) to non-synonymous (dN) substitutions was estimated by using the Nei–Gojobori method (http://www.hiv.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html) (Korber, 2000).

3. Results

3.1 Loss of *GHRL* and *MBOAT4* in the platypus genome

Previous research reported wholesale loss of genes required for gastric function (Ordonez et al., 2008), which prompted us to look at a set of genes central to metabolic control involving both stomach and pancreas.

Interestingly, at first glance the ghrelin gene (*GHRL*), encoding a key hormone expressed in stomach and pancreatic ε cells, seemed to be missing in publicly available platypus genome databases (Ensembl, NCBI and UCSC) (Warren et al., 2008). BLAST search with the full opossum *GHRL* sequences in the platypus genome including trace archives also failed to reveal the existence of *GHRL* in the platypus genome. A general NCBI BLASTN (Altschul et al., 1997) search with opossum *GHRL* cDNA sequences produced matches across vertebrate species, including human (*Homo sapiens*), mouse (*Mus musculus*), Tasmanian devil (*Sarcophilus harrisii*), wild boar (*Sus scrofa*) and other species with the notable exception of platypus. We also searched the transcriptome databases ViroBLAST (Deng et al., 2007) and ISA modules (Brawand et al.) to search for transcript signatures of a monotreme ghrelin gene but no *GHRL* transcripts were detected.

To exclude poor assembly as a reason for the absence of *GHRL* sequences, we investigated the genomic region surrounding the *GHRL*.

Synteny analysis of the region containing *GHRL* showed strong conservation among vertebrate species (Fig. 1A). Analysis of eight genes flanking *GHRL* in human (*IRAK2*, *TATDN2*, *SEC13*, *ATP2B2*, *SLC6A11*, *HRH1*, *VGLL4* and *TAMM41*) revealed that these genes are present in platypus but located on different ultra contigs and chromosomes: *IRAK2* lies on Contig31581, *TATDN2* and *TAMM41* on Ultra529, *SEC13* on Ultra602, *ATP2B2*, *SLC6A11*, *HRH1* and *VGLL4* is located on chrX1 (Fig. 1A).

In humans *GHRL* is situated between *TATDN2* and *SEC13*. In platypus these two genes are located on different contigs (see Fig. 1A). To address the possibility that this region has undergone rearrangements and that the *GHRL* has been translocated elsewhere in the genome, we performed a series of degenerate PCRs to identify any *GHRL* related sequences in platypus.

Degenerate primers were designed to bind to the most conserved regions identified by multiple coding sequences alignments of *GHRL* in several vertebrate species (see Materials and Methods, Table 1 and Supplementary Fig. 1). These primers successfully amplified *GHRL* in chicken, opossum and mouse genomic DNA, but no band was observed in platypus (Fig. 2A and Table 2) and echidna (Supplementary Fig. 5). This suggests that the *GHRL* has been lost or mutated beyond recognition in monotremes.

The only known function of GOAT is to catalyze the *O*-n-octanoylation of ghrelin, which lead us to speculate that the loss of functional ghrelin removed the need for GOAT activity and, therefore, rendered the gene encoding GOAT (*MBOAT4*) obsolete. Hence, we investigated the presence of *MBOAT4* in the platypus genome following the same strategy as used for *GHRL*.

Again searches, as described above, did not reveal any sequence in the platypus genome that matched *MBOAT4* in other species. A general NCBI BLASTN (Altschul et al., 1997) search with opossum *MBOAT4* cDNA sequences produced matches across vertebrate species, including human (*Homo sapiens*), mouse (*Mus musculus*), Tasmanian devil (*Sarcophilus harrisii*), wild boar (*Sus scrofa*) and other species but not platypus.

We next investigated the region surrounding the *MBOAT4* in other mammals.

Synteny analysis of the region harboring *MBOAT4* showed conservation among vertebrate species (Fig. 1B). Analysis of three genes flanking *MBOAT4* in human, namely, *TMEM66*, *LEPROTL1* and *DCTN6* are located on two different super contigs (Fig. 1B).

We then used degenerate PCRs to identify any *MBOAT4* like sequences in platypus.

Degenerate primers were designed based on multiple coding sequences (cds) alignment of *MBOAT4* in several vertebrate species (see Materials and Methods, Table 1 and Supplementary Fig. 1). Again, primers amplified from genomic DNA of opossum and mouse but not platypus or echidna (Fig. 2B). This suggests that *MBOAT4* along with *GHRL* has been lost or substantially changed in monotremes.

3.2 Identification and characterization of platypus ghrelin receptor *GHSR*

The *GHSR* has two exons and one intron in eutherian mammals. We identified partial sequence of this gene in the platypus genome database. The platypus genomic and predicted protein coding sequences available in ENSEMBL (Transcript ID: ENSOANG00000016050) and NCBI (Gene ID: 100092085) contains gaps at the 5' end of the first exon and lacks the entire second exon of platypus *GHSR*. Partial exon1 sequences is present on contig 104977. BLAST search using human *GHSR* sequences in UCSC identified sequence homologous to exon 2 on the platypus UltraContig 395:458,994-459,299. The same contig also harbors the flanking genes (*FNDC3B* Ultra395:12,310-364,361 and *TNFSF10* Ultra395:425299-444108) (Supplementary Fig. 2). In order to investigate whether the two contigs containing *GHSR* sequence are linked, we performed genomic PCR with primer sets spanning exon 1 and exon 2. This amplified a 4.5 kb product showing that platypus has an intact ghrelin receptor gene (Supplementary Fig. 3). Multiple alignment of platypus *GHSR* with other species shows a high level of sequence conservation and no sign of positive selection (Averages of all pairwise comparisons: ds = 1.1177, dn = 0.2748, ds/dn = 7.8032, ps/pn = 4.5716 (Korber, 2000)). Next we investigated the expression pattern of this receptor in platypus. RT-PCR revealed strong expression of platypus *GHSR* in brain, intestine and kidney, with weaker expression in the heart and stomach (Fig. 3). This expression pattern is similar to that seen in other mammals, which predominantly express *GHSR* in brain, but also in peripheral organs,

including heart, lung, liver, kidney, pancreas, stomach and intestine (Gnanapavan et al., 2002; Guan et al., 1997).

Alignment of the partial amino acid sequences of platypus GHS-R 1a with other amniote species shows that this gene is conserved through evolution including platypus (Fig. 4B). Sequence comparison between human and platypus showed a high level of conservation with 81% amino acid identity. Moreover, residues that are important for ghrelin to bind and exert biological activities are: Cys^{116}, Gln^{120}, Glu^{124}, Glu^{187}, Cry^{198}, Trp^{276}, Phe^{279}, Arg^{283}, Phe^{286} and Asn^{305} in human GHS-R 1a (Holst et al., 2006; Holst et al., 2009; Ueda et al., 2011). These residues are identical in platypus GHS-R 1a (Fig. 4A), implying that although ghrelin and GOAT have been lost in platypus, the *GHSR* remains intact, and it encodes GHS-R 1a with a conserved ghrelin binding site.

Alternative splicing of human *GHSR* generates two isoforms: GHS-R 1a is produced by the splicing of exon 1 and exon 2 in the primary transcript, in contrast, GHS-R 1b mRNA is created by the termination of transcription at part of intron 1 (Howard et al., 1996) (Fig. 5A). We tried to investigate whether these isoforms exist in platypus. PCR with primer pairs spanning exon 1 and 5' end of intron 1 (Fig. 5B and Supplementary Fig. 4A) produced a 470 bp band in platypus genomic DNA, but not cDNA (Supplementary Fig. 4B). It is therefore likely that the GHS-R 1b splice variant does not exist in platypus.

4. Discussion

The ghrelin pathway is evolutionarily highly conserved among vertebrate species, but little is known about this pathway in egg-laying mammals (platypus and echidna). Monotremes are a fascinating group of mammals to study the evolution of metabolic control as they have a dramatically different gastric system compared to other mammalian species and have lost

many digestion-related genes (Ordonez et al., 2008). Here we investigated three genes, *GHRL*, *MBOAT4* and *GHSR* of the brain-gut axis ghrelin pathway in monotremes.

Surprisingly our experiments suggest that in monotremes the *GHRL* and *MBOAT4* have been lost or substantially mutated whereas *GHSR* is present, conserved and expressed as in other mammals.

Ghrelin acetylation appears to be catalyzed exclusively by the enzyme GOAT and no other substrates of GOAT are known (Yang et al., 2008b; Ohgusu et al., 2009). In this context the attrition of the *MBOAT4* may have been the result of the loss of ghrelin.

These results raise the possibility that the lineage specific loss of ghrelin and GOAT in monotremes may be related to the loss of gastric function or has been replaced by redundant ligands acting through the ghrelin receptor.

4.1 Is the loss of ghrelin related to the monotreme specific changes in the gastrointestinal tract?

Ghrelin is mainly released by the P/D_1 cell in humans and X/A-like cell in rodents in the oxyntic gland of the gastric mucosa (Date et al., 2000). The platypus stomach is anatomically and physiologically different from other mammalian species. Many genes involved in digestion and stomach function have been lost or are inactivated (Ordonez et al., 2008). The platypus stomach has been described as “glandless” with only Brunner’s glands present (Griffiths, 1978). The loss of ghrelin raises questions about the existence of the ghrelin producing X/A-cells in platypus. Although the platypus stomach is generally described as glandless there is not specific information about glands other than Brunner’s glands that have been described in (Griffiths, 1978).

Another gene that is typically expressed in X/A-cells, Nesfatin-1 (*NUCB2*), is present in the platypus genome. This leaves open the possibility that X/A cells are present or that *NUCB2* has a function in other cells in the stomach.

Ghrelin has also been described as a marker gene for the newly discovered ε cells in the human pancreas (Wierup et al., 2004), but there is some controversy over whether ghrelin is exclusively expressed in ε cells in the human pancreas as its expression has also been reported in human and rat pancreatic α and β cells (Date et al., 2002; Volante et al., 2002). In contrast to the stomach, the monotreme pancreas anatomy is similar to other mammals. Previous histological work ((Yamada et al., 1990) and Tsend-Ayush *et al.* (unpublished observation) has identified endocrine islets of Langerhans containing α, β, δ and PP cells in echidna and platypus, but it is unknown if pancreatic ε cells exist in monotremes.

4.2 The loss of ghrelin raises question about metabolic control in monotremes

The effects of ghrelin deletion and overexpression have been investigated in a number of vertebrates but this is the first time the evolutionary loss of this gene has been reported.

Ghrelin was initially discovered as a GH releasing and appetite regulating hormone, however the effects of ghrelin vary among species. Ghrelin enhances GH release in most mammals, birds, frog and fish (reviewed by (Kaiya et al., 2012)). However, the effects after loss of ghrelin on development differ between species. For example, while zebrafish lacking ghrelin show impaired growth (Li et al., 2009), *Ghrl* KO mice have a normal growth rate, body composition and size (De Smet et al., 2006; Sun et al., 2003; Sun et al., 2008; Sun et al., 2004). The effects of ghrelin on appetite regulation vary as well. In humans and rat the presence of ghrelin stimulates appetite, whereas there is no effect on other mammals (for example, mice and pigs) and in chicken feeding is inhibited (reviewed by (Kaiya et al.,

2012)). In mice the lack of ghrelin leads to metabolic changes including an enhanced insulin response and faster clearance rate of glucose when fed on a high fat diet (Dezaki et al., 2006). Also, these mice tend to have a lower body fat mass and reduced respiratory quotient when fed on a high-fat diet, indicating increased fat oxidation (Wortley et al., 2004). At this stage we can only speculate as to the effect of lack of ghrelin on metabolism on monotreme metabolism but it is possible that the communication mechanism between hunger signaling and insulin responsiveness through ghrelin may be have been lost as a consequence of the loss of peptic digestion.

4.3 Is there another ligand(s) acting via GHS-R 1a in monotremes?

In contrast to the loss of *GHRL* and *MBOAT4* we did identify the platypus ortholog *GHSR*. This raises the possibility of ghrelin independent signaling through this receptor in monotremes. The human GHS-R 1a appears to have a high degree of ghrelin-independent constitutive signaling activity (around 50% of its maximum activity) (Holst et al., 2003). Early studies have suggested an endogenous inverse agonist that regulates the receptor by decreasing the constitutive activity (Holst et al., 2003). GHS-R 1a residues involved in constitutive activation: Glu^{124}, Ala^{204}, Phe^{279} and Arg^{281} are conserved in platypus, indicating that the platypus ghrelin receptor may also exhibit a degree of constitutive activity.

Ghrelin, GOAT and GHS-R 1a knockout mice have revealed numerous roles for this signaling system (reviewed in (Kang et al., 2011; Albarran-Zeckler et al., 2011)) including neuroprotection, learning and memory, modulation of dopamine signaling, and thymopoeisis. Presumably a loss of such wide-ranging activities would be detrimental to monotremes, which raises the possibility that other ligands may be acting via GHS-R 1a in monotremes. The existence of other ligands is also directly indicated by several studies (Furness et al., 2011; Holst et al., 2003; Pfluger et al., 2008). It is worth noticing that in eutheria, although GHSR

1a is present and has conserved functions in the CNS, there is no ghrelin in the spinal cord or most other parts of the CNS, and ghrelin released from stomach cannot cross the blood-brain barrier to enter the spinal cord. Therefore, similar to the situation of monotremes, the conserved function and location of spinal cord GHSR1a, without exposure to ghrelin suggests the presence of an endogenous, but unknown ligand in eutheria (Furness et al., 2011). The discovery of ghrelin, was through the construction of a cell line stably expressing GHS-R 1a to monitor the intercellular Ca^{2+} level that were induced by rat tissue extracts. The highest activity was observed in the stomach extracts containing ghrelin (Kojima et al., 1999), leaving the possibility that other ligands with lower activity may have been overlooked.

Inconsistent and unexpectedly mild phenotypes of ghrelin pathway gene knockouts in animal models (reviewed in (Kaiya et al., 2012))(De Smet et al., 2006; Sun et al., 2003; Sun et al., 2008; Sun et al., 2004) further supports redundancy in this pathway. Interestingly, simultaneous KO of both *Ghrl* and its receptor enhanced the phenotype of single gene-deficient mice with decreased body weight and fat, increased energy expenditure and locomotor activity on a standard diet.

In conclusion, we report data suggesting for the first time the loss or attrition of *GHRL* and *MBOAT4* in monotremes. It is currently unknown what the consequences are in terms of growth, appetite, metabolic control and glucose regulation in monotremes. The presence of the ghrelin receptor in monotremes and mild and variable phenotype in animal models may indicate that the ghrelin receptor exerts its function independently of ghrelin or possibly via activation by another ligand. Monotremes are a unique system to further study the action of the ghrelin receptor in the absence of functional ghrelin.

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References

Albarran-Zeckler, R.G., Sun, Y., Smith, R.G., 2011. Physiological roles revealed by ghrelin and ghrelin receptor deficient mice. Peptides. 32, 2229-2235.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., et al., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

Banks, W.A., Tschop, M., Robinson, S.M., Heiman, M.L., 2002. Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. J. Pharmacol. Exp. Ther. 302, 822-827.

Brawand, D., Soumillon, M., Necsulea, A., Julien, P., Csárdi, G., Harrigan, P., et al., Comparative transcriptome analyses reveal the evolution of gene expression levels in mammalian organs (http://www2.unil.ch/cgi-bin/cgiwrap/wwwcbg/devel/isaweb.py).

Chow, K.B., Sun, J., Chu, K.M., Tai Cheung, W., Cheng, C.H., Wise, H., 2012. The truncated ghrelin receptor polypeptide (GHS-R1b) is localized in the endoplasmic reticulum where it forms heterodimers with ghrelin receptors (GHS-R1a) to attenuate their cell surface expression. Mol. Cell. Endocrinol. 348, 247-254.

Date, Y., Kojima, M., Hosoda, H., Sawaguchi, A., Mondal, M.S., Suganuma, T., et al., 2000. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology. 141, 4255-4261.

Date, Y., Nakazato, M., Hashiguchi, S., Dezaki, K., Mondal, M.S., Hosoda, H., et al., 2002. Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. Diabetes. 51, 124-129.

De Smet, B., Depoortere, I., Moechars, D., Swennen, Q., Moreaux, B., Cryns, K., et al., 2006. Energy homeostasis and gastric emptying in ghrelin knockout mice. J. Pharmacol. Exp. Ther. 316, 431-439.

Deng, W., Nickle, D.C., Learn, G.H., Maust, B., Mullins, J.I., 2007. ViroBLAST: a stand-alone BLAST web server for flexible queries of multiple databases and user's datasets. Bioinformatics. 23, 2334-2336.

Dezaki, K., Sone, H., Koizumi, M., Nakata, M., Kakei, M., Nagai, H., et al., 2006. Blockade of pancreatic islet-derived ghrelin enhances insulin secretion to prevent high-fat diet-induced glucose intolerance. Diabetes. 55, 3486-3493.

Furness, J.B., Hunne, B., Matsuda, N., Yin, L., Russo, D., Kato, I., et al., 2011. Investigation of the presence of ghrelin in the central nervous system of the rat and mouse. Neuroscience. 193, 1-9.

Gnanapavan, S., Kola, B., Bustin, S.A., Morris, D.G., McGee, P., Fairclough, P., et al., 2002. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J. Clin. Endocrinol. Metab. 87, 2988.

Griffiths, M., 1978. Food and feeding habits: digestive organs and digestion, in: The Biology of Monotremes, Academic Press, New York, pp. 77-81.

Guan, X.M., Yu, H., Palyha, O.C., McKee, K.K., Feighner, S.D., Sirinathsinghji, D.J., et al., 1997. Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res. 48, 23-29.

Gutierrez, J.A., Solenberg, P.J., Perkins, D.R., Willency, J.A., Knierman, M.D., Jin, Z., et al., 2008. Ghrelin octanoylation mediated by an orphan lipid transferase. Proc. Natl. Acad. Sci. U. S. A. 105, 6320-6325.

Holst, B., Cygankiewicz, A., Jensen, T.H., Ankersen, M., Schwartz, T.W., 2003. High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist. Mol. Endocrinol. 17, 2201-2210.
Holst, B., Frimurer, T.M., Mokrosinski, J., Halkjaer, T., Cullberg, K.B., Underwood, C.R., et al., 2009. Overlapping Binding Site for the Endogenous Agonist, Small-Molecule Agonists, and Ago-allosteric Modulators on the Ghrelin Receptor. Mol. Pharmacol. 75, 44-59.
Holst, B., Lang, M., Brandt, E., Bach, A., Howard, A., Frimurer, T.M., et al., 2006. Ghrelin Receptor Inverse Agonists: Identification of an Active Peptide Core and Its Interaction Epitopes on the Receptor. Mol. Pharmacol. 70, 936-946.
Howard, A.D., Feighner, S.D., Cully, D.F., Arena, J.P., Liberator, P.A., Rosenblum, C.I., et al., 1996. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science. 273, 974-977.
Kaiya, H., Kangawa, K., Miyazato, M., 2012. What is the general action of ghrelin for vertebrates? - Comparisons of ghrelin's effects across vertebrates. Gen. Comp. Endocrinol.
Kaiya, H., Miyazato, M., Kangawa, K., Peter, R.E., Unniappan, S., 2008. Ghrelin: a multifunctional hormone in non-mammalian vertebrates. Comp. Biochem. Physiol., A: Mol. Integr. Physiol. 149, 109-128.
Kaiya, H., Mori, T., Miyazato, M., Kangawa, K., 2009. Ghrelin receptor (GHS-R)-like receptor and its genomic organisation in rainbow trout, Oncorhynchus mykiss. Comp. Biochem. Physiol., A: Mol. Integr. Physiol. 153, 438-450.
Kang, K., Zmuda, E., Sleeman, M.W., 2011. Physiological role of ghrelin as revealed by the ghrelin and GOAT knockout mice. Peptides. 32, 2236-2241.
Kirchner, H., Gutierrez, J.A., Solenberg, P.J., Pfluger, P.T., Czyzyk, T.A., Willency, J.A., et al., 2009. GOAT links dietary lipids with the endocrine control of energy balance. Nat. Med. 15, 741-745.
Kirchner, H., Heppner, K.M., Tschop, M.H., 2012. The role of ghrelin in the control of energy balance. Handb. Exp. Pharmacol. 161-184.
Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., Kangawa, K., 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 402, 656-660.
Kojima, M., Kangawa, K., 2005. Ghrelin: structure and function. Physiol. Rev. 85, 495-522.
Kojima, M., Kangawa, K., 2008. Structure and function of ghrelin. Results Probl. Cell Differ. 46, 89-115.
Korber, B., 2000. HIV Signature and Sequence Variation Analysis, in: A.G. Rodrigo, G.H. Learn, (Eds.), Computational Analysis of HIV Molecular Sequences, Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 55-72.
Krause, W.J., 1971. Brunner's glands of the duckbilled platypus (Ornithorhynchus anatinus). Am. J. Anat. 132, 147-165.
Krause, W.J., Leeson, C.R., 1974. The gastric mucosa of two monotremes: the duck-billed platypus and echidna. J. Morphol. 142, 285-299.
Leung, P.K., Chow, K.B., Lau, P.N., Chu, K.M., Chan, C.B., Cheng, C.H., et al., 2007. The truncated ghrelin receptor polypeptide (GHS-R1b) acts as a dominant-negative mutant of the ghrelin receptor. Cell. Signal. 19, 1011-1022.
Li, X., He, J., Hu, W., Yin, Z., 2009. The essential role of endogenous ghrelin in growth hormone expression during zebrafish adenohypophysis development. Endocrinology. 150, 2767-2774.
Ohgusu, H., Shirouzu, K., Nakamura, Y., Nakashima, Y., Ida, T., Sato, T., et al., 2009. Ghrelin O-acyltransferase (GOAT) has a preference for n-hexanoyl-CoA over n-octanoyl-CoA as an acyl donor. Biochem. Biophys. Res. Commun. 386, 153-158.

Ordonez, G.R., Hillier, L.W., Warren, W.C., Grutzner, F., Lopez-Otin, C., Puente, X.S., 2008. Loss of genes implicated in gastric function during platypus evolution. Genome Biol. 9, R81.

Pfluger, P.T., Kirchner, H., Gunnel, S., Schrott, B., Perez-Tilve, D., Fu, S., et al., 2008. Simultaneous deletion of ghrelin and its receptor increases motor activity and energy expenditure. Am J Physiol Gastrointest Liver Physiol. 294, G610-618.

Sakata, I., Yang, J., Lee, C.E., Osborne-Lawrence, S., Rovinsky, S.A., Elmquist, J.K., et al., 2009. Colocalization of ghrelin O-acyltransferase and ghrelin in gastric mucosal cells. Am. J. Physiol. Endocrinol. Metabol. 297, E134-141.

Stengel, A., Goebel, M., Yakubov, I., Wang, L., Witcher, D., Coskun, T., et al., 2009. Identification and characterization of nesfatin-1 immunoreactivity in endocrine cell types of the rat gastric oxyntic mucosa. Endocrinology. 150, 232-238.

Strauss, W.M., 2001. Preparation of Genomic DNA from Mammalian Tissue, in: Current Protocols in Molecular Biology, John Wiley & Sons, Inc.

Sun, Y., Ahmed, S., Smith, R.G., 2003. Deletion of ghrelin impairs neither growth nor appetite. Mol. Cell. Biol. 23, 7973-7981.

Sun, Y., Butte, N.F., Garcia, J.M., Smith, R.G., 2008. Characterization of adult ghrelin and ghrelin receptor knockout mice under positive and negative energy balance. Endocrinology. 149, 843-850.

Sun, Y., Wang, P., Zheng, H., Smith, R.G., 2004. Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proc. Natl. Acad. Sci. U. S. A. 101, 4679-4684.

Tanaka, M., Hayashida, Y., Iguchi, T., Nakao, N., Nakai, N., Nakashima, K., 2001. Organization of the mouse ghrelin gene and promoter: occurrence of a short noncoding first exon. Endocrinology. 142, 3697-3700.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Ueda, T., Matsuura, B., Miyake, T., Furukawa, S., Abe, M., Hiasa, Y., et al., 2011. Mutational analysis of predicted extracellular domains of human growth hormone secretagogue receptor 1a. Regul. Pept. 166, 28-35.

Volante, M., Allia, E., Gugliotta, P., Funaro, A., Broglio, F., Deghenghi, R., et al., 2002. Expression of ghrelin and of the GH secretagogue receptor by pancreatic islet cells and related endocrine tumors. J. Clin. Endocrinol. Metab. 87, 1300-1308.

Warren, W.C., Hillier, L.W., Marshall Graves, J.A., Birney, E., Ponting, C.P., Grutzner, F., et al., 2008. Genome analysis of the platypus reveals unique signatures of evolution. Nature. 453, 175-183.

Wierup, N., Svensson, H., Mulder, H., Sundler, F., 2002. The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. Regul. Pept. 107, 63-69.

Wierup, N., Yang, S., McEvilly, R.J., Mulder, H., Sundler, F., 2004. Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells. J. Histochem. Cytochem. 52, 301-310.

Wortley, K.E., Anderson, K.D., Garcia, K., Murray, J.D., Malinova, L., Liu, R., et al., 2004. Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. Proc. Natl. Acad. Sci. U. S. A. 101, 8227-8232.

Yamada, J., Krause, W.J., Edwin, N., Mochizuki, T., Yanaihara, N., 1990. A survey of endocrine cells in the pancreas of the echidna (Tachyglossus aculeatus) with special reference to pancreatic motilin cells. J. Anat. 171, 223-231.

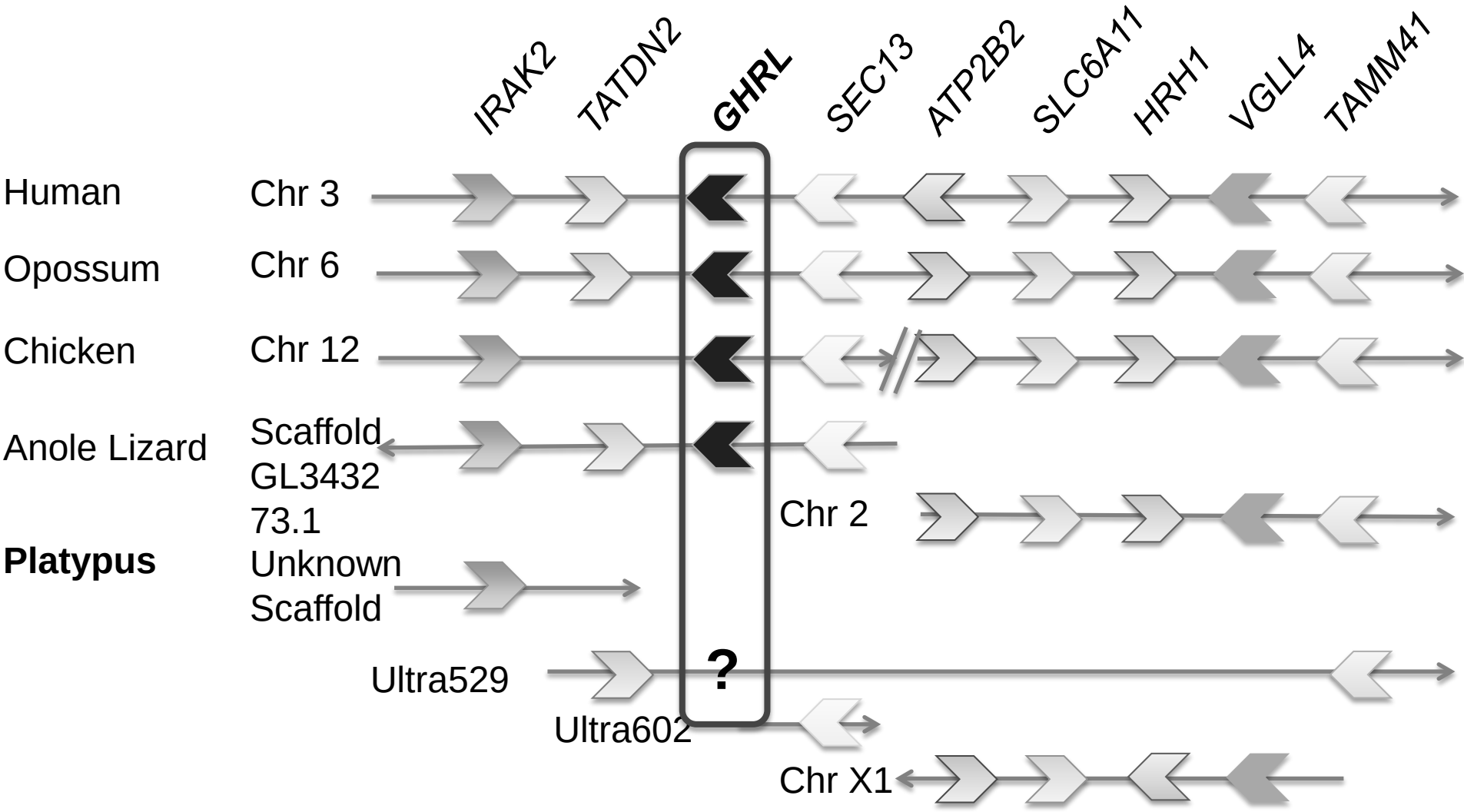
Yang, J., Brown, M.S., Liang, G., Grishin, N.V., Goldstein, J.L., 2008a. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell. 132, 387-396.

Yang, J., Zhao, T.J., Goldstein, J.L., Brown, M.S., 2008b. Inhibition of ghrelin O-acyltransferase (GOAT) by octanoylated pentapeptides. Proc. Natl. Acad. Sci. U. S. A. 105, 10750-10755.

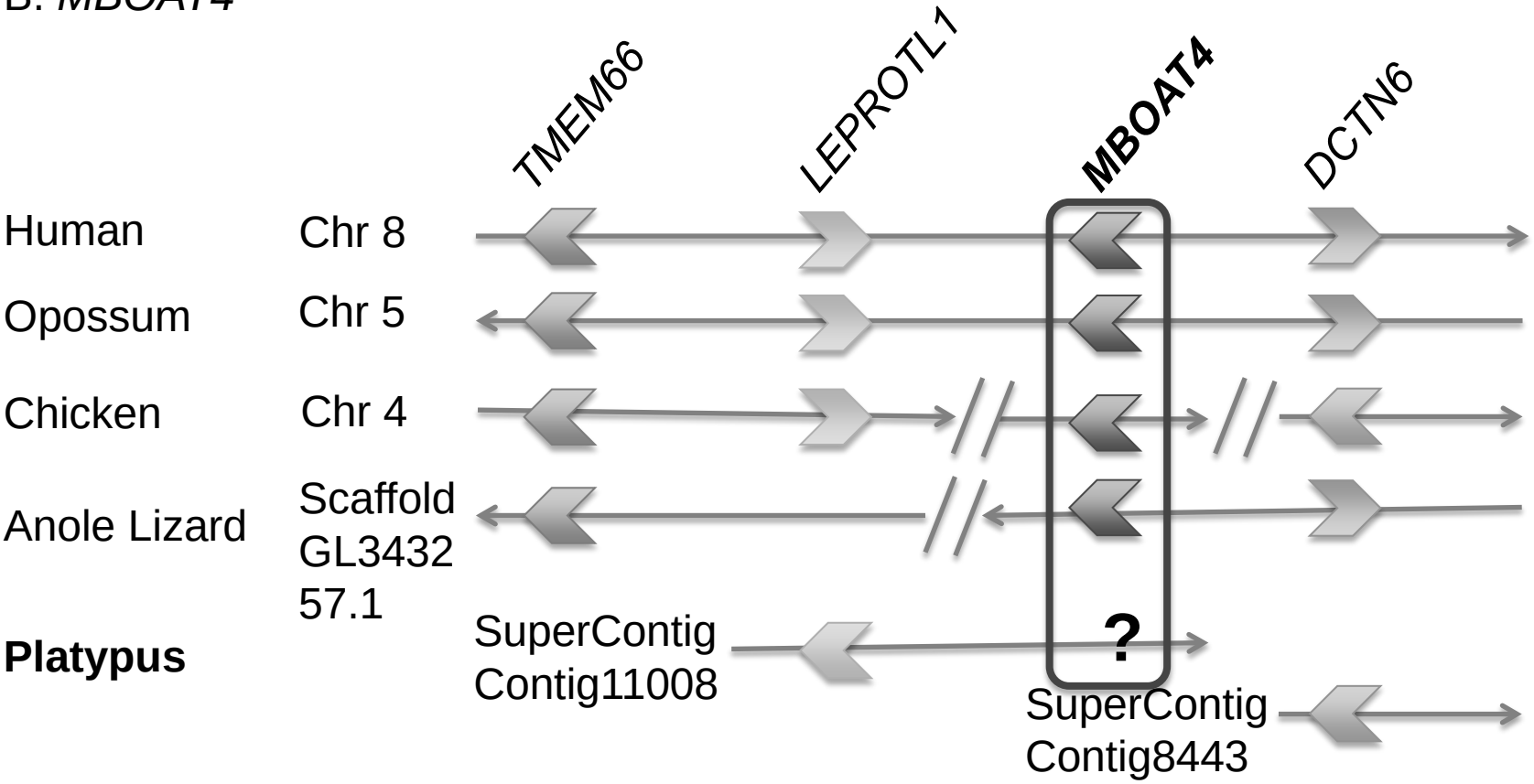
Zhang, J.V., Ren, P.G., Avsian-Kretchmer, O., Luo, C.W., Rauch, R., Klein, C., et al., 2005. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science. 310, 996-999.

Figure 1

A. *GHRL*



B. *MBOAT4*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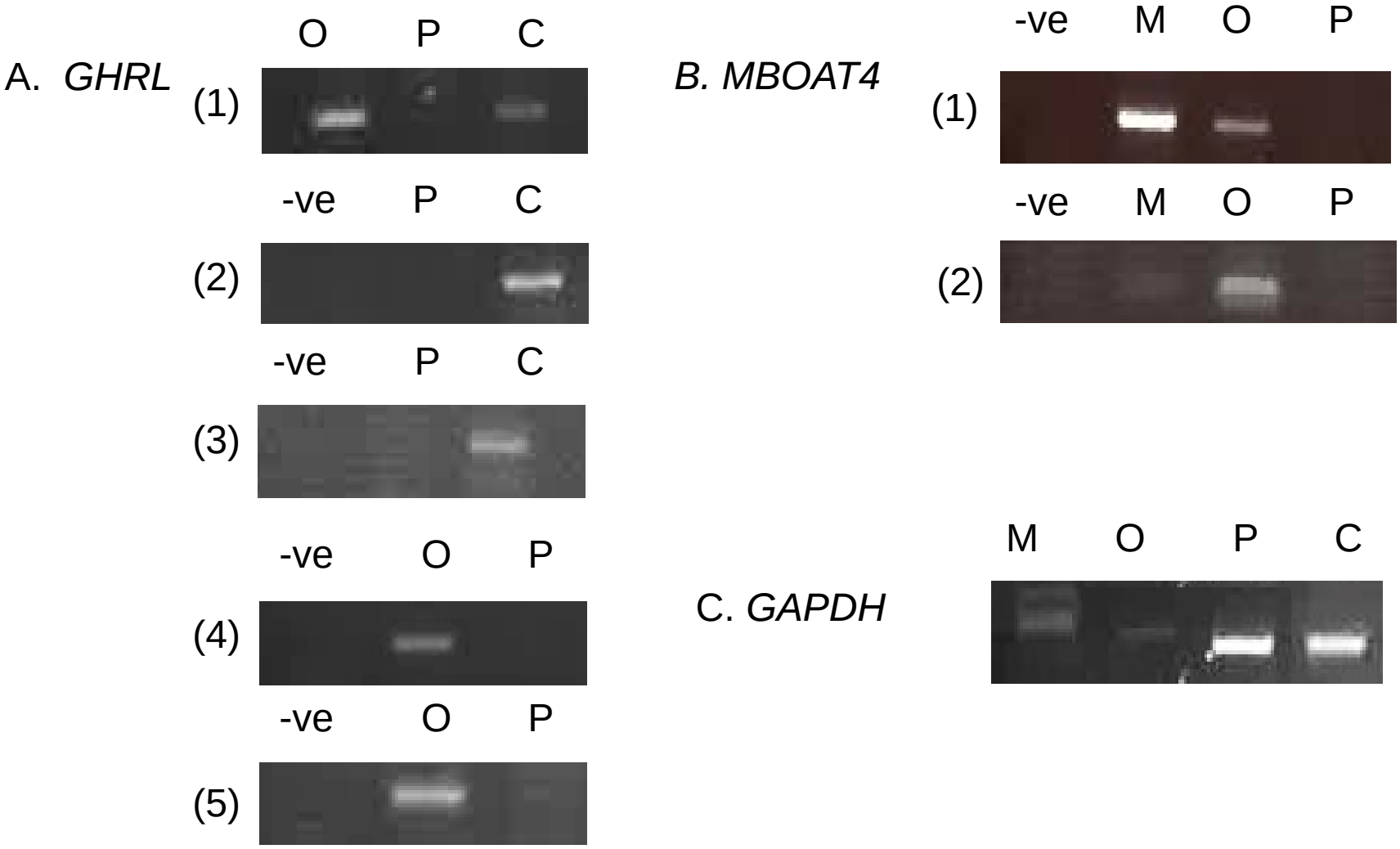
LEPROTL1
MBOAT4
DCTN6
Human
Chr 8
Opossum
Chr 5
Chicken
Chr 4
Anole Lizard
Scaffold GL343257.1
Platypus
SuperContig Contig11008
?
SuperContig Contig8443

Figure 2


A. GHRL
O P C
(1)
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(2)
-ve P C
(3)
-ve O P
(4)
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(5)
B. MBOAT4
-ve M O P
(1)
-ve M O P
(2)
C. GAPDH
M O P C

Figure 3

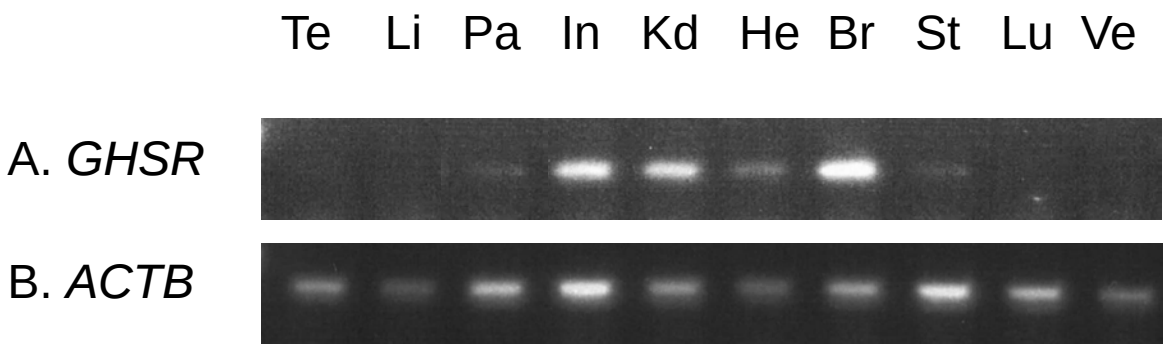
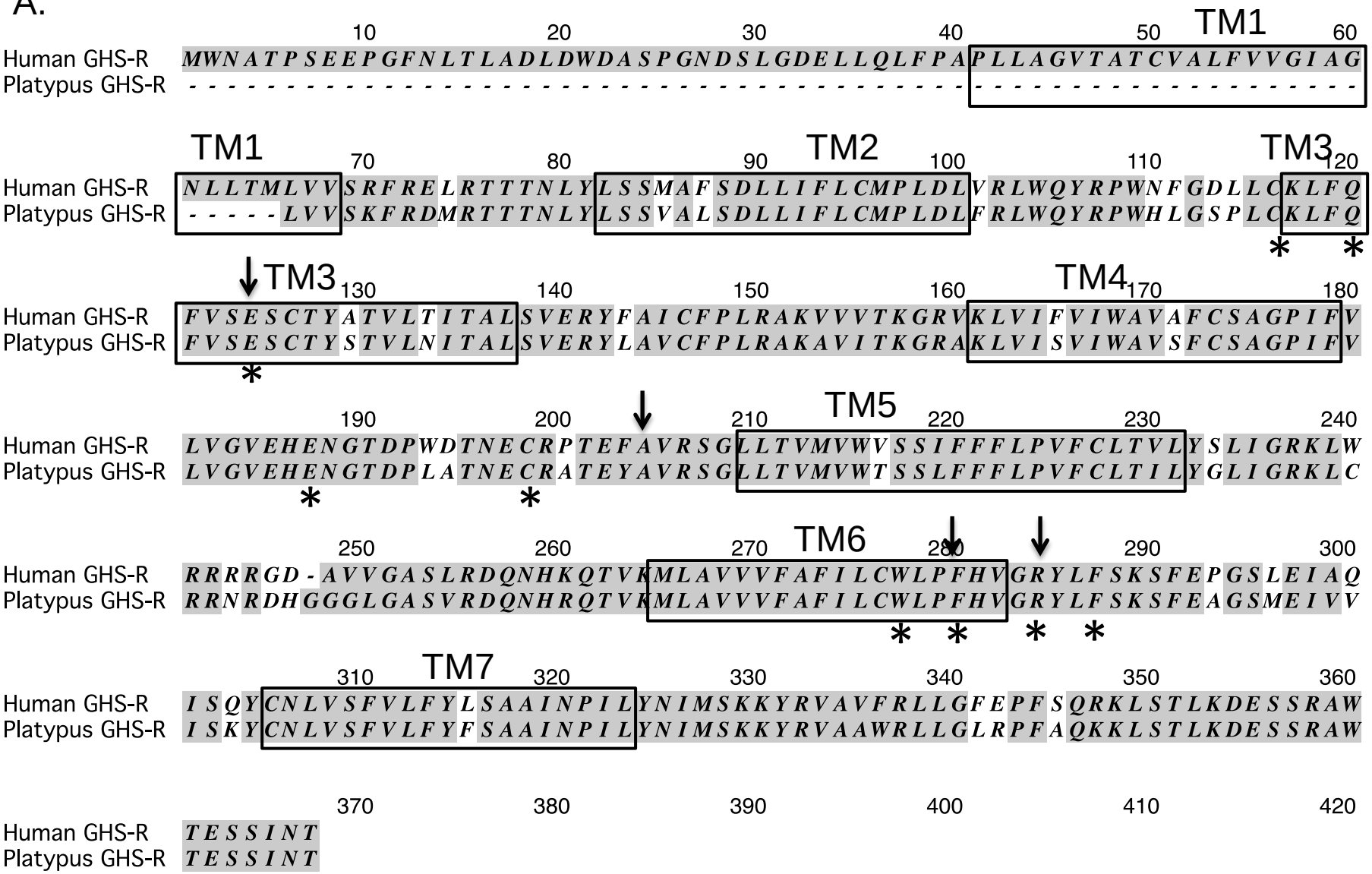


Figure 4

A.


TM1
TM1
TM2
TM3
TM3
TM4
TM5
TM6
TM7
Human GHS-R
Platypus GHS-R
Human GHS-R
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Human GHS-R
Platypus GHS-R
Human GHS-R
Platypus GHS-R
Human GHS-R
Platypus GHS-R
Human GHS-R
Platypus GHS-R
Human GHS-R
Platypus GHS-R

B.

Method: Neighbor Joining; Bootstrap (1000 reps); tie breaking = Systematic
Distance: Poisson-correction
Gaps distributed proportionally

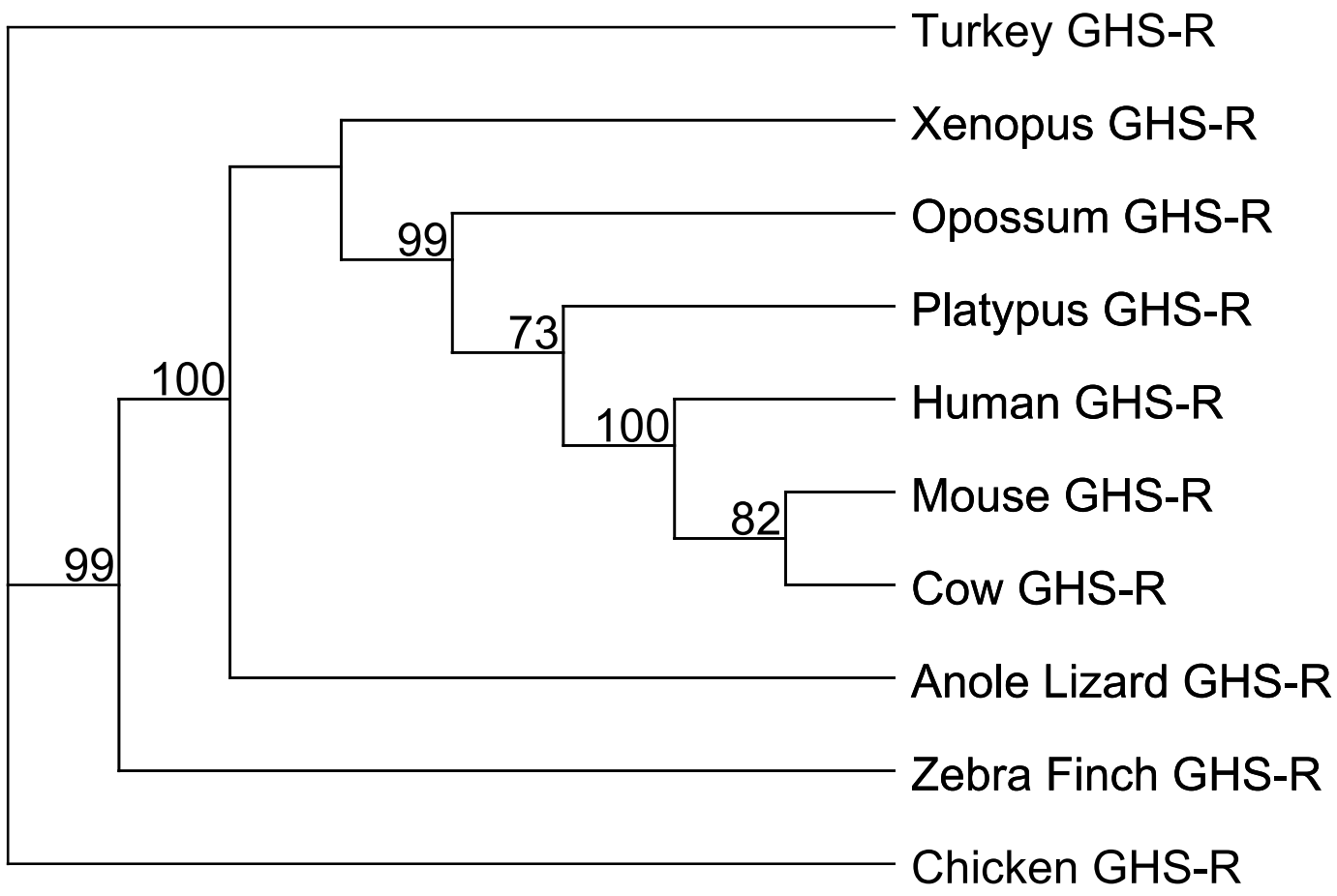
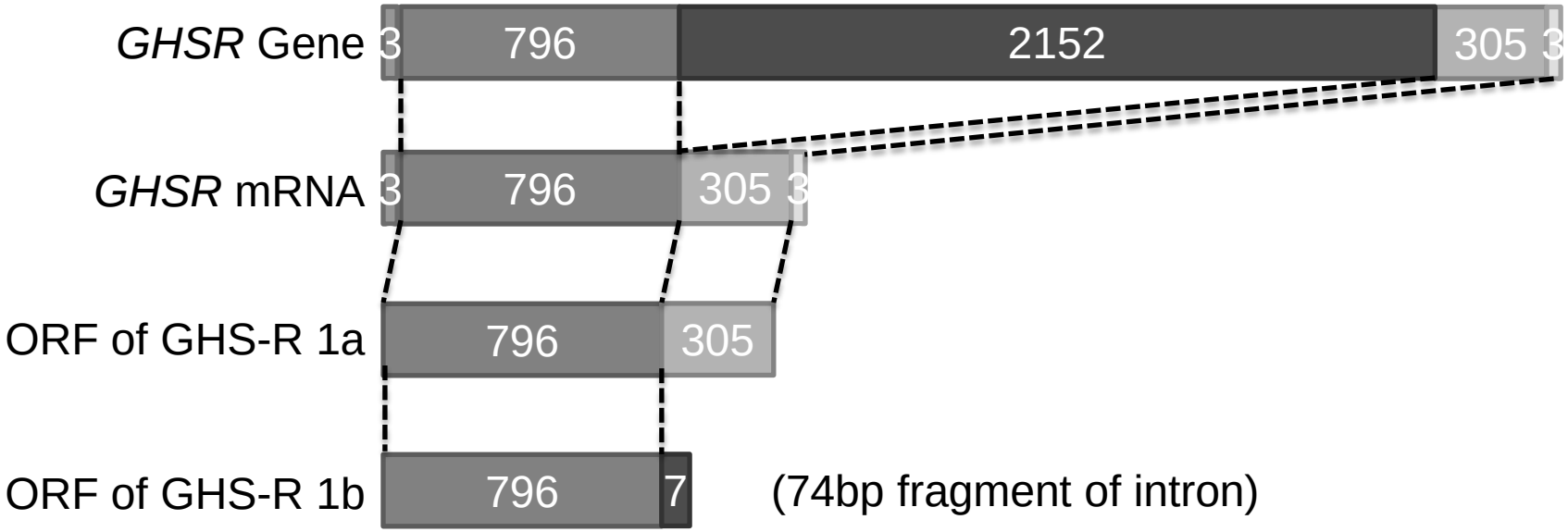


Figure 5

A. Human *GHSR*



B. Platypus *GHSR*